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**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

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Inhibition of CD137 for tumor therapy

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# Inhibition of CD137 for tumour therapy

**Abstract**

CD137 (ILA, 4-1BB), a member of the TNF receptor family regulates proliferation, differentiation and survival of immune cells (Schwarz et al., 1993; Lotz et al., 1996). CD137 can inhibit T cell proliferation and induce programmed cell death in T cells leading to a downregulation of T cell activity (Schwarz et al., 1996).

We have discovered that CD137 is expressed ectopically by chronic lymphocytic leukemia (CLL) cells and other tumors while corresponding non-malignant tissues do not express CD137. Expression of CD137 provides the tumors with growth advantages and mechanisms to blunt the host anti-tumor immune response.

Neutralization or downregulation of CD137 expression on tumor cells has been shown to allow their elimination by immune cells. Strategies aimed at inhibition CD137 expression or activity are proposed as new therapeutic methods for treatment of tumors.

CD137 or CD137 ligand binding molecules may be exploited to downregulate harmful immune responses.

**Introduction**

The cytokine receptor CD137 is a member of the tumor necrosis factor receptor family. CD137 is expressed by activated T and B lymphocytes and expression in primary cells is strictly activation dependent (Schwarz et al., 1995). The gene for human CD137 resides on chromosome 1p36, in a cluster of related genes, and this chromosomal region is associated with mutations in several malignancies (Schwarz et al., 1997).

Crosslinking of CD137 costimulates proliferation of T lymphocytes (Goodwin et al., 1993; Pollock et al., 1993; Schwarz et al., 1996), and CD137 ligand expressed by B lymphocytes costimulates T cell proliferation synergistically with B7 (DeBenedette et al., 1995).

While agonistic antibodies and the ligand to CD137 enhance lymphocyte activation, CD137 protein has the opposite effect. It inhibits proliferation of activated T lymphocytes and induces

programmed cell death. These T cell-inhibitory activities of CD137 require immobilisation of the protein, arguing for transmission of a signal through the ligand/coreceptor (Schwarz et al., 1996; Michel et al., 1999).

The known human CD137 ligand is expressed constitutively by monocytes and its expression is inducible in T lymphocytes (Alderson et al., 1994). Monocytes are activated by immobilized CD137 protein and their survival is profoundly prolonged by CD137. (Langstein et al., 1998; Langstein et al., 1999a). CD137 also induces proliferation in peripheral monocytes (Langstein et al., 1999b). Macrophage colony-stimulating factor (M-CSF) is essential for the proliferative and survival-enhancing activities of CD137 (Langstein et al., 1999a; Langstein et al., 1999b).

Signaling through CD137 ligand has also been demonstrated in B cells where it enhances proliferation and immunoglobulin synthesis. This occurs at interactions of B cells with CD137-expressing T cells or follicular dendritic cells (Pauly et al., 2002). It was postulated that similarily to the CD40 receptor/ligand system, which mediates T cell help to B cells after first antigen encounter, the CD137 receptor/ligand system may mediate co-stimulation of B cells by FDC during affinity maturation (Pauly et al., 2002).

Soluble forms of CD137 are generated by differential splicing and are selectively expressed by activated T cells (Michel et al., 1998). Soluble CD137 is antagonistic to membrane-bound or immobilized CD137, and levels of soluble CD137 correlate with activation induced cell death in T cells (DeBenedette et al., 1995; Hurtado et al., 1995; Michel et al., 2000).

**Novelty of the invention**

CD137 is expressed by tumors as a neoantigen and provides protection from the host immune response. Specifically, CD137 induces apoptosis in cytotoxic immune cells. In addition, CD137 expression leads to TGF-β secretion by the tumor cells which further inhibit anti-tumour immune responses.

These facts were not known before and discovered in our study for the fist time. They lead to the following potential therapy for tumors: Neutralization of CD137 expressed by tumors or

inhibition of CD137 expression by tumors will enable the immune system of the patient to reduce or eliminate the tumor mass.

A further implication of these findings is that recombinant CD137 protein, or analogs thereof as well as CD137 expression constructs can be used to downregulate immune responses in autoimmune disease, allergy and asthma and transplantation.

**Claims**

1, Diagnosis of CD137-expressing tumors using CD137-specific antibodies, peptides or organic small molecules or nucleotide sequences specific for CD137.

2, Neutralization of CD137 using CD137-specific antibodies, peptides or organic small molecules for therapy of CD137-expressing tumors.

3, Inhibition of CD137 expression using CD137-specific antisense oligonucleotides, small interfering RNAs or CD137 antisense expression vectors or recombinant viruses for therapy of CD137-expressing tumors.

4, Neutralization of CD137 ligand(s) using CD137 ligand-specific antibodies, peptides or organic small molecules for tumor therapy.

5, Inhibition of CD137 ligand expression using CD137 ligand-specific antisense oligonucleotides, small interfering RNAs or CD137 ligand antisense expression vectors or recombinant viruses for tumor therapy.

6, Agonistic anti-CD137 ligand antibodies for treatment of conditions characterized by overactive immune reactions, such as autoimmune disease, allergy and asthma or for the inhibition of undesired immune responses i.e. in organ transplantation.

7, Recombinant CD137 protein or analogs, including peptides and small molecules for treatment of conditions characterized by overactive immune reactions such as autoimmune disease, allergy and asthma and transplantation or for the inhibition of undesired immune responses i.e. in organ transplantation.

8, CD137 expression vectors or CD137-expressing viral vectors for treatment of conditions characterized by overactive immune reactions such as autoimmune disease, allergy and asthma and transplantation or for the inhibition of undesired immune responses i.e. in organ transplantation.

**Summary**

This patent application describes the ectopic expression of CD137 by tumors and its use by tumors to evade the host immune response. Neutralization of CD137 on tumors or inhibition of its expression by tumors are proposed and claimed as new ways of tumor therapy. The ability of CD137 to downregulate immune responses can be utilized for treatment of diseases characterized by overactive immune reactions.

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# CD137 can be expressed by tumor cells as a neoantigen and prolongs cell survival and provides protection from lymphokine activated killer cell lysis

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Abbreviations: antigen presenting cells: APC; chronic lymhocytic leukemia, CLL; lymphokine activated killer, LAK; sCD137,

## INTRODUCTION

Most tumor patients develop an immune response against their tumor. But often this immune response is not sufficient for tumor eradication. Tumor cells develop and are being selected for their successful development of defense mechanisms against the host immune response. One category of these defense mechanisms is the ectopic expression of immunoregulatory molecules, which inhibit the host immune response. Many glioblastomas express TGF-β, a potent antiinflammatory molecule and the neutralization of TGF-β can enable the immune system to eliminate the tumor (Jachimczak et al., 1993). A similar function has been shown for the disintegrin MIA, which protects melanomas from lysis by cytotoxic T cells (Apfel et al., 2002). Another immune regulatory molecule is CD95 ligand, which is expressed by cytotoxic T cells and natural killer cells in order to induce programmed cell death in target cells. CD95 ligand can be expressed by normal tissues to maintain an immune privileged status. Its ectopic expression is exploited by hepatomas and other tumors which use CD95 ligand to kill tumor-infiltrating immune cells (Strand et al., 1996; Ferrone et al., 2000; O'Connel et al., 1999).

CD137 is a member of the TNF receptor family and is expressed by activated T cells (Schwarz et al., 1993; Schwarz et al., 1995; Lotz et al., 1996). Expression in primary cells is strictly activation dependent. Constitutive expression is only found in transformed cells (Schwarz et al., 1995). Crosslinking of CD137 delivers a costimulatory signal to T cells. CD137 ligand and agonistic anti-CD137 antibodies enhance T cell proliferation and cytotoxicity in vitro, and anti-tumor and anti-transplant immune responses in vivo (Schwarz et al., 1996; Maus et al., 2002; Melero et al., 1997, Shuford et al., 1997).

CD137 ligand is expressed on the surface of antigen presenting cells (APC) and upon crosslinking can deliver activating signals to APC. Immobilized CD137 protein induces activation of peripheral monocytes with enhanced expression of proinflammatory cytokines and activation markers and a reduction of the antiinflammatory cytokine IL-10 (Langstein et al., 1998). CD137 also prolongs survival and induces proliferation in monocytes (Langstein et al., 1999a; Langstein et al., 1999b). Through its bidirectional signaling capacity, the CD137 receptor/ligand system can exert potent immunostimulatory activities by activating T cells and APC simultaneously.

Signaling through CD137 ligand has also been demonstrated in B cells where it enhances proliferation and immunoglobulin synthesis. This occurs at interactions of B cells with CD137-expressing T cells or follicular dendritic cells (Pauly et al., 2002). It was postulated that similarily to the CD40 receptor/ligand system, which mediates T cell help to B cells after first antigen encounter, the CD137 receptor/ligand system may mediate co-stimulation of B cells by FDC during affinity maturation (Pauly et al., 2002).

A ligand for CD137 is also expressed by T cells and in contrast to the CD137 receptor the ligand delivers inhibitory signals to T cells. Immobilized CD137 protein inhibits T cell proliferation and induces cell death by apoptosis (Schwarz et al., 1996; Michel et al., 1999).

Soluble forms of CD137 are generated by differential splicing and are selectively expressed by activated T cells (Michel et al., 1998). Soluble CD137 is antagonistic to membrane-bound or immobilized CD137, and levels of soluble CD137 correlate with activation induced cell death in T cells (DeBenedette et al., 1995; Hurtado et al., 1995; Michel et al., 2000).

Here we show that CD137 is expressed by malignant B cells especially chronic lymphocytic leukemia (CLL) cells, but not by normal B cells. CD137 expression provides tumor cells with the advantage of prolonged survival. However, proliferation of CLL cells was found to be not affected by CD137. Further, CD137 expression protects tumor cells from elimination by lymphokine activated killer (LAK) cells by inducing apoptosis in LAK cells. Further, crosslinking of CD137 ligand by CD137, leading to secretion of TGF-β, was identified as an additional mechanism of CD137-mediated protection.

## MATERIAL AND METHODS

*Reagents.*

The plasmid CMV-ILA-SEN (CIS) was constructed by inserting the human CD137 (ILA) cDNA into the eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA). The cDNA was excised from the cloning vector pSPORT by the restriction enzymes EcoRI and HaeIII, which cut in the vector polycloning site 5' to the CD137 cDNA and in the 3' untranslated region of the CD137 cDNA at position 921, respectively. The cDNA was inserted into Bluescript (Stratagene, San Diego, CA) via EcoRI and SmaI, yielding plasmid ILA-3'del. From there the CD137 cDNA was inserted into pcDNA3 by the restriction sites NotI and HindIII. The plasmids RSV-ILA-SEN (RIS) and RSV-ILA-AS (RIA) are based on the eukaryotic expression vector pRC/RSV (Invitrogen, San Diego, CA). The CD137 cDNA fragment from the plasmid ILA-3'del was inserted into pRSV in its sense (RIS) orientation by NotI and HindIII, and in the antisense orientation (RIA) by HindIII, XbaI, respectively. Sequencing confirmed the correct reading frames and sequence of the plasmids.

CD137-Fc protein was purified from supernatants of stably transfected CHO cells by protein G sepharose, as described previously (Schwarz et al., 1996). Human IgG1 Fc protein was

purchased from Accurate Chemical and Scientific Corporation, (Westbury, NY, USA). Anti-CD137 antibody (clone BBK-2) and its isotype control, MOPC21 were obtained from Bioscource (Ratingen, Germany) and Sigma (Deisenhofen, Germany), respectively.

Anti TGF-β antibody (clone MAB240) was obtained from R&D Systems, Wiesbaden, Germany.

*Cells and cell culture.*

Jurkat, Raji and K562, CHO and COS cells were obtained from ECACC (Salisbury, UK).

Human peripheral blood mononuclear cells (PBMC) were isolated from fresh blood obtained from healthy volunteers. 50 ml of whole blood were collected and 10 U/ml of heparin were added immediately. The blood was spun for 20 min at 2500 rpm (1000 g), the pellet was resuspeded in 120 ml of RPMI (without serum) and 5 U/ml of heparine were added. 30 ml of that were layered onto 15 ml of Histopaque (Sigma, Deisenhofen, Germany). After centrifugation at 1800 rpm (450 g) for 35 min the cells from the boundary layer of each tube were collected and resuspended in 25 ml RPMI and were washed in 10 ml RPMI, and were finally resuspended at $2-3 \times 10^6$ cells/ml in RPMI 10% FCS.

B-CLL cells were isolated from peripheral blood of patients by Histopaque gradient density centrifugation as above. Removal of T cells by negative selection using anti-CD3 beads resulted 96 to 99 % pure B-CLL cell populations.

B cells were isolated from PBMC. Fractions with enriched B cells were collected by elutriation [Andreesen et al., 1990] and contained between 60% and 80% B cells as estimated

by CD19 expression. In a second step using magnetic anti-CD19-beads (Miltenyi, Bergisch-Gladbach) the B cells were purified to > 95%.

*Cell proliferation*

Proliferation of cell populations was determined in a 96-well microtiter plate. $10^6$ CLL cells per well in a 100 µl volume were pulsed during the last 24 hours of culture with 0.5 µCi $^3$H-thymidine and harvested and evaluated on the TopCount microplate scintillation counter Packard (Meriden, CT, USA). Each data point is mean of five independent measurements and depicted as mean ± standard deviation.

*Flow cytometry analysis*

Cells were analyzed using a FACS-Calibur (Becton Dickinson, Mountain View, CA) and Cellquest software. One million cells were used per condition. Cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS, 2% FCS), resuspended in 50 µl FACS buffer and stained with PE-conjugated anti-CD137 antibody, (dilution 1:50, clone 4B4-1; Ancell, Bayport, MN), PE-conjugated isotype control antibody (dilution 1:10, Dianova, Hamburg, Germany) and/or PE-conjugated anti-CD19 antibody (dilution 1:25, clone UCHT1, dako, Hamburg Germany) for 30 min at 4$^0$ C. After two washes cells were analyzed by flow cytometry.

*Immunohistochemistry.*

Frozen tissue sections were fixed with 2 % paraformaldehyde for 10 min. Endogenous peroxidases were inactivated by 6.5 % hydrogen peroxide in methanol for 15 min. Unspecific staining was blocked by 3 % dry milk in PBS for 30 min. 2 µg/ml of anti-CD137 (clone BBK-2, Bioscource, Ratingen, Germany) or an isotype control antibody (MOPC 21, Sigma,

Deisenhofen, Germany) in 3 % dry milk were added overnight. The entire procedure was carried out at RT and after each step the samples were washed three times with PBS. Staining was performed at 37°C with the ABC kit (Dako, Hamburg, Germany) using diaminobenzidine as substrate. Tissue sections were stained with hematoxylin and embedded in Entellan (Merck, Darmstadt, Germany).

*Confocal microscopy*

Cells were spun onto a microscope slide and dried for half an hour at room temperature, fixed in ice-cold aceton and dried again for half an hour. After that cells were rehydrated in PBS for 15 min at room temperature and blocked for half an hour with 50 µl PBS, 3 % BSA. Double staining was carried out with FITC-conjugated anti-CD19 antibody (dilution 1:10, clone HD37, DAKO, Hamburg, Germany) and biotinylated anti-CD137 antibody (dilution 1:50, clone 4B4-1, Ancell, Bayport, MN) for 1 h at 37°C in the dark. FITC-conjugated murine IgG1 (dilution 1:10, Dianova, Hamburg, Germany) and biotinylated mouse IgG1 (dilution 1:10, Dako, Hamburg, Germany) were used as isotype control antibodies, respectively. After a 1 h incubation at room temperature the slides were washed with PBS and coverd with streptavidin-Cy3 for 1 h at room temperature in the dark. After three washes cell nuclei were stained with 4 µg/ml Hoechst 33342 (Sigma, Deisenhofen, Germany for half an hour at room temperature. The cells were washed and mounted with Mobi Glow (MoBiTec). The slides were stored in the dark at 4°C.

*Cytotoxicity assays*

$10^6$ target cells per ml were washed twice with PBS, 5 % FCS (PBS-F) and were loaded with 20 µg/ml of CalceinAM (Molecular Probes, Leiden, The Netherlands) for 20 min at 37°C. Cells were washed twice and $10^4$ loaded target cells per well in 100 µl PBS-F were incubated

in 96 well plates with varying numbers of LAK cells for 4 h at 37°C. LAK cells were generated by activating PBMC with IL-2 (100 ng/ml) for 3 days. Values for spontaneous release ($FL_{sp.}$) were obtained by incubating loaded target cells without LAK cells, and total release ($FL_{tot.}$) was determined by lysing target cells with lysis buffer (50 mM sodium borate, 0.1% Triton–X 100, pH 9.0). Cells were removed by centrifugation and released Calcein was quantified in a Fluoroscan (Titertek, Fluoroscan II, Meckenheim, Germany) with filter settings at extinction 2, emission 2. The percentage of lysis was calculated according to the following formula: $(FL_{assay} - FL_{sp.}) / (FL_{tot.} - FL_{sp.}) \times 100 = \%$ cytotoxicity (Lichtenfels et al., 1994).

*Apoptosis assays*

Induction of apoptosis was determined by measuring annexin V and prodidium iodine staining of cells using the Annexin-V-FLUOS staining Kit (Roche, Mannheim, Germany) according to the manufacture's instructions.

*Transfection*

COS, CHO, K562 and Jurkat cells were transfected using the Lipofectamin/Plus-method (Invitrogen, Groningen, The Netherlands). At the day before transfection COS and CHO cells were seeded in a T25 tissue culture flask at $5 \times 10^5$ cells and cultured overnight in DMEM supplemented with 10% FCS. At the day of transfection, the cells were washed in serum-free DMEM and taken up in 2 ml serum-free DMEM. 3 µg DNA were diluted in 115 µl s serum-free DMEM and 10 µl of Plus-Reagent were added. After 15 min at room temperature 15 µl Lipofectamin Reagent diluted in 110 µl serum-free DMEM were gently pipetted to the DNA-Plus-mixture. Incubation for 15 min at room temperature was performed to allow complex formation. Then the DNA-Lipofektamin-Plus-solution was added to the cells. 10 % FCS was added after 3 h at 37°C.

Jurkat and K562 cells were seeded in a 24 well plate in 200 µl serum-free RPMI at 1 x $10^6$ cells. 3 or 4 µg of DNA were diluted in 70 µl of serum-free RPMI for Jurkat or 4 K562 cells, respectively. 5 µl of Plus Reagent were added and the mixture was incubated for 15 min at room temperature. 5 µl of Lipofektamin were added to the mixture and the DNA-Lipofectamin-Plus-Solution was incubated for 15 min at room temperature for complex formation and afterwards added to the cells. 3 h later 1 ml RPMI and 10% FCS were added.

Raji cells were transfected by the DMRIE-C-method: Rajis were washed in OPTIMEM (Invitrogen). 3 µl of DMRIE-C (Invitrogen) were diluted in 125 µl OPTIMEM in an 24 well-plate. 0.75 µg DNA diluted in 125 µl OPTIMEM were added and the mixture was incubated for 45 min to allow complex formation. The DMRIE-C-DNA-Mix was added to 50 µl containing 5 x $10^5$ cells. After 4 h incubation at 37°C and 5% $CO_2$ 1 ml RPMI and 10% FCS were added.

Cells were used in experiments two days after transfection.

*ELISA*

Antibody pairs suitable for IL-10 and TGF-ß1 ELISAs were purchased from R&D Systems (Wiesbaden, Germany). Puffers were made according to the manufacturer's instructions. 96 well ELISA plates were coated over night with the capture antibody at a 1:180 dilution. The plates were washed three times with washing puffer and blocked with blocking puffer for an hour at 37°C. After three washes samples and standards were added an incubated for 1 h at 37°C. In the case of TGF-ß1 the samples were activated with 1/5 volume of 1 N HCl for 10 min and neutralized with 1/6 volume of 1.2 N NaOH/0.5 M HEPES. IL-10 samples were used without prior treatment. The plates were washed again and with a 1:180 dilution of the detection antibody for 1 h at 37°C. After three washes a 2 µg/ml ABTS-solution in ABTS-puffer (Roche Diagnostics, Mannheim, Germany) was added and the plates were analyzed in

an ELISA-reader. Cytokine concentrations were determined in triplicate and are expressed as mean ± standard deviation.

## RESULTS

### CD137 is expressed as a neoantigen on CLL cells

Expression of CD137 is inducible in T cells by activation with mitogens and CD137 levels are higher in CD8-positive cells than in CD4-positive ones (Fig. 1). No expression of CD137 protein could be detected on human peripheral B cells of more than 10 different donors, though several activation conditions were tested, including PHA, PMA + calcium ionophore, anti-IgM and anti-CD40 + IL-4 (Fig. 1). However, CD137 mRNA has been detected in primary activated B cells (Schwarz et al., 1995), indicating that expression of CD137 in B cells is suppressed at the posttranscriptional level.

B-CLL cells isolated from the peripheral blood of CLL patients expressed CD137 protein after activation by PHA or PMA + calcium ionophore (Fig. 2A). CD137 expression was detectable on a subset of CLL cells from all 14 patients tested, and the number of CD137-positve cells ranged from 2.7 to 58.3 % (Fig. 2B).

Double staining for CD137 and the B cell-specific marker CD19 and subsequent analysis by confocal microscopy confirmed that the CD137-expressing cells were in fact B cells (Fig. 2C). Colocalization of CD137 and CD19 on B cells was observed with cells from all of the eight patients analyzed. Interestingly, in about half of the CD137-positive CLL cells the CD137 protein was not evenly distributed over the cell surface but was clustered in spots (Fig. 2C).

In addition to CLL cells constitutive expression of CD137 was detected in a on a highly malignant anaplastic B cell lymphoma, located on the wall of the sinus cavity. The majority of the tumor cells expressed the B cell marker CD20. No T cells could be detected within the tumor based on CD3 staining (Fig. 2D).

**Immobilized CD137 prolongs survival in CLL cells.**

During the transformation process tumor cells may start to express genes, which are silent in the parental differentiated cells. Many of these neoantigens provide the tumor cells with a growth or selection advantage. The selective expression of CD137 on malignat but not on primary B cells implied a similar role for CD137. CD137 ligand is constitutively expressed by B cells and upon crosslinking by CD137 costimulates B cell proliferation (Pauly et al., 2002). Therefore, CD137 expression could allow B-CLL cells to enhance their survival and growth in an autocrine manner.

The ability of cell surface expressed CD137 to crosslink its ligand was simulated by coating a fusion protein consisting of the extracellular domain of CD137 and the constant domain of human immunoglobulin G1 (CD137-Fc) onto cell culture dishes. Untreated and Fc protein coated plates were used as negative controls. Coating was performed with a solution of 10 µg/ml CD137-Fc protein in PBS at 4°C overnight. Fc protein was used an equimolar concentration of 5 µg/ml.

Immobilized CD137-Fc significantly prolonged B-CLL cell survival while the Fc control protein had no effect (Fig. 3). These data indicate that immobilized CD137 protein crosslinks a ligand or coreceptor on the CLL cells, which delivers the survival signal.

The in vitro survival of CLL cells was donor-dependent and the cells from the six patients, which were investigated had half-lifes between 2 and 12 days (Table 2). Most cells were dead after 12 – 20 days when cultured on uncoated or Fc coated plates. CLL cells grown on immobilized CD137-Fc survived significantly longer. The maximum effect of CD137 was visible at day 8 when the number of live cells in all six CLL cell population was 10 – 30% higher that in the controls. From five out of the six CLL cell populations, cells continued to survive on immobilized CD137-Fc after all cells in the controls had died off.

Table 2

| CLL patient | Fc | CD137-Fc | x-fold survival |
|---|---|---|---|
| HE | 16,0 ± 1,8 | 34,0 ± 9,2 | 2.12 |
| MA | 12,4 ± 1,0 | 18,8 ± 3,5 | 1.52 |
| MÜ | 73,1 ± 6,9 | 84,4 ± 0,9 | 1.16 |
| PI | 26,4 ± 3,4 | 36,5 ± 1,0 | 1.38 |
| TR | 32,9 ± 5,6 | 71,0 ± 5,1 | 2.16 |
| DE | 45,1 ± 1,2 | 71,7 ± 2,3 | 1.58 |

Table 2: Influence of CD137 on in vitro survival of CLL cells.

$10^7$ B-CLL cells were cultured on 10 µg/ml immobilized CD137-Fc protein or an equimolar concentration of Fc protein (5 µg/ml). The numbers of live cells were determined on day 8 by trypan blue staining. Four random fields were counted and are expressed as percentage of live cells, based on the number of live cells at the beginning of the experiment. Stated are mean ± standard deviation. x-fold survival represents the ratio of life cells in CD137-Fc vs. Fc coated plates.

In order to determine a potential effect of CD137 on the growth rate, B-CLL cells were cultured on immobilized CD137 as described above for 3 or 8 days, and labelled with $^3$H-thymidine for the last 16 h of culture. In four independent experiments with CLL cells from

four different donors up to 2-fold higher proliferation was measured with CD137-Fc treated compared to untreated or Fc treated CLL cells (not shown). However, when incorporated radioactivity was adjusted for the larger number of live cells in the CD137-coated wells, no effect of CD137 on CLL cell proliferation remained.

These data demonstrate that CD137 prolongs the survival of B-CLL cells but does not influence proliferation. They further imply that CLL cells may express CD137 in order to provide each other with survival signals in a paracrine manner.

**Expression of CD137 protects cells from lysis by lymphokine activated killer cells.**

Next we investigated whether CD137 expression by CLL cells also influences the host immune response against the tumor cells. For assessing the effects of CD137 expression we used cells transfected with a CD137 expression vector or the empty control vector as target cells in cytotoxicity assays. Human PBMC activated for three days with 100 ng/ml of IL-2 were used as lymphokine activated killer (LAK) cells. CD137-transfected cells were lysed at significantly lower rates than the control transfected cells at target:effector ratios ranging from 1:1 to 1:50 (Fig. 4A). The protective effect of CD137 was not cell type specific. Rather it was obtained in experiments using the the B cell line Raji, the T cell line Jurkat, and the chronic myelogenous leukemia line K562 as targets (Fig. 4A).

Since Jurkat, K562 and Raji cells express CD137 constitutively it was possible to perform also the reverse experiment and to test whether reduction of CD137 expression resulted in an increased LAK-mediated lysis. Cells, which were transfected with a CD137 antisense vector were indeed lysed at higher rates than cells transfected with the empty control vector (Fig. 4A). Again, this result was obtained with all three cell lines.

Successful modulation of CD137 expression by transfection of CD137 sense and antisense constructs was verified by flow cytometry and is exemplary shown for Raji and K562 cells (Fig. 4B).

In addition to modulating expression of CD137 on target cells we also tested whether neutralization of CD137 by specific antibodies would influence susceptibility to lysis. No constitutive expression of CD137 is detectable on COS cells with the available anti-CD137 antibodies. Incubation of CD137-transfected COS cells with anti-CD137 antibody for 16 h increased the rate of lysis up to threefold, while the antibody did not affect lysis of untransfected COS cells (Fig 4C). Similarily, preincubation with anti-CD137 antibody for 16 h enhanced lysis of Raji cells, which express CD137 constitutively, and enhanced lysis correlated with the concentration of the neutralizing anti-CD137 antibody (Fig 4C).

These experiments involving modulation of CD137 expression and functional inhibition of CD137 by specific antibodies clearly demonstrate that CD137 levels correlate with the protective effect against LAK cell-mediated lysis.

**Induction apoptosis by CD137 is not responsible for reduced LAK cytotoxicity.**

A potential mechanism for protection against lysis by LAK cells could be induction of cell death. CD137 has been previously been shown to induce cell death by apoptosis in T cells (Schwarz et al., 1996; Michel at al., 1999).

In order to investigate whether LAK cells are being be driven into apoptosis by CD137-expressing target cells, LAK cells were cocultured with CD137-transfected and mock

transfected COS cells, which do not express CD137 constitutively. 5 - 24 h later the LAK cells were analysed by flow cytometry using propidium iodine and annexinV staining.

The numbers of late apoptoptic and necrotic cells were higher in the presence of CD137 (Table 3). The same result was obtained when LAK cells were incubated with other CD137-expressing cells. Compared to mock transfection, CD137 transfection of Raji and K562 cells increased LAK cell apoptosis only from 3.3 to 4.2%, or from 8.1 to 10.4%, respectively.

Table 3

| COS cells transfected with | target: effector ratio | live AnnexinV$^-$ PI$^-$ | early apoptotic AnnexinV$^+$ PI$^-$ | late apoptotic AnnexinV$^+$ PI$^+$ | necrotic AnnexinV$^-$ PI$^+$ |
|---|---|---|---|---|---|
| --- | --- | 75.2 | 6.2 | 16.6 | 2.0 |
| PcDNA3 | 1: 1 | 70.3 | 9.4 | 17.5 | 2.8 |
| | 1: 2,5 | 76.6 | 5.9 | 15.4 | 2.2 |
| | 1: 10 | 77.8 | 5.5 | 14.9 | 1.9 |
| CD137 | 1: 1 | 69.8 | 7.1 | 19.9 | 3.2 |
| | 1: 2,5 | 71.1 | 6.2 | 18.7 | 4.0 |
| | 1: 10 | 74.3 | 5.5 | 16.8 | 3.5 |

Table 3: Influence of CD137 on LAK cell death.

PBMC were activated by 100 ng/ml of IL-2 for 3 days to generate LAK cells. COS cells, which had been transfected with the CD137 expression vector CIS (CD137), or the empty expression vector (pcDNA3) were used as target cells. Effector and target cells were incubated for 24 h at indicated ratios. Percentages of live (Annexin V$^-$, PE$^-$), early apoptotic (Annexin V$^+$, PE$^-$), late apoptotic (Annexin V$^+$, PE$^+$) and necrotic effector cells (Annexin V$^+$, PE$^+$) were determined by flow cytometry. Identical results were obtained in two independent experiments.

**CD137 induces expression of TGF-β.**

Having demonstrated that CD137 expression by target cells induces apoptosis in LAK cells we felt that this may not be the sole mechanism of CD137-mediated protection. Firstly, the degree of apoptosis induction was overall small. And the change in LAK cell apoptosis after transfection of CD137 sense or antisense vectors was smaller that the change in target cytotoxicity. Further, CD137-induced apoptosis is a slow process peaking around day 3 (Schwarz et al., 1996; Michel at al., 1999) while the cytotoxicity assay lasted only 4 h.

Besides induction of apoptosis in immune cells, secretion of antiinflammatory cytokines is a powerful mechanism of tumor cells to evade immune surveillance. The antiinflammatory activities of IL-10 and TGF-β and their utilization by tumor cells are well documented (Dumont and Arteaga, 2000; Akhurst and Derynck, 2001; Pasche, 2001). Therefore, concentrations of TGF-β and IL-10 were measured in supernatants of LAK cells after exposure to CD137. Coculture of CD137 expressing cells had no noticable effect on TGF-β or IL-10 secretion by LAK cells (not shown).

However, CD137 regulates cytokine expression by the target cells. Raji cells express TGF-β and IL-10 constitutively. Transfection with the CD137 expression vector further increased levels of TGF-β, while reduction of constitutive CD137 expression by transfection of the CD137 antisense vector reduced TGF-β secretion by Raji cells (Fig 5). Levels of IL-10 remained unchanged (Fig. 5).

K562 cells also express TGF-β constitutively but no IL-10 was detectable in their supernatants. Similarly to Raji cells, levels of TGF-β were increased or decreased by CD137

sense or antisense transfection, respectively (Fig. 5). In Jurkat cells no secretion of TGF-β could be detected while levels of IL-10 were independent of CD137 expression (Fig. 5).

Modulation of TGF-β expression by CD137 sense and antisense transfection was consistent for the target cells used. IL-10 secretion seemed to be rather independent of CD137 expression.

**CD137-induced TGF-β mediates protection against lysis by LAK cells**

We have shown that the amount of CD137 expression correlates (1) with the degree of protection of target cells from LAK-mediated cytotoxicity, and, (2) with TGF-β levels secreted by the target cells. This implied that TGF-β induction was the mechanism responsible for CD137-mediated protection. In order to verify this hypothesis neutralizing anti-TGF-β antibodies were added to the target cells from 6 h after transfection up to the cytotoxicity assay at day 2. Neutralization of secreted TGF-β in the 2 day period prior to the cytotoxicity assay rendered the target cells more susceptible to LAK lysis (Fig. 6). Target cells were thoroughly washed before being used in the cytotoxicity assay to avoid carry-over of anti-TGF-β antibodies into the assay. Also, no TGF-β could be detected in the supernatants of the cytotoxicity assay at the end of the experiment (not shown). Further, neutralizing anti-TGF-β antibodies had no effect when added directly to the LAK assay, instead of being added to the target cells prior to the assay (not shown). This indicates that TGF-β does not mediate protection from LAK lysis by inhibiting LAK cell activity. Rather, it seems that CD137-induced TGF-β secretion makes target cells more resistant to lysis by LAK cells. A possible mechanism would be induction of members of the bcl-2 family, which have been widely documented to raise the threshold of cell death induction (Adams and Cory; 1998).

## DISCUSSION

Tumor cells express neoantigens as a result of chance mutations. Some of these neoantigens provide the tumor cells with growth and / or survival advantages and become selected and enriched in the tumor cell population.

This study establishes that immobilized CD137 prolongs CLL cell survival in vitro. This effect is not large but was observed with cells from all six patients tested and was significant in five of them. No effect of CD137 on CLL cell proliferation could be observed. It remains an open question whether CD137 affects survival and proliferation of CLL cells in vivo and to what extent it contributes to tumor growth.

Crosslinking of CD137 ligand through immobilized CD137 protein or CD137 expressed on transfected cells enhances proliferation and immunoglobulin synthesis of primary B cells (Pollok et al., 1994; Pauly et al., 2002). Under physiological conditions this costimulation would occur during interactions of T cells with B cells or follicular dendritic cells (DeBenedette et al., 1997; Pauly et al., 2002). The ectopic expression of CD137 could enable CLL cells to imitate these interactions and to provide each other mutually with survival signals in a paracrine manner. Functional signaling of CD137 is implied by its clustering into cell surface structures, which are compatible with microdomains. Similar clustering or assembly to rafts has been observed for other costimulatory molecules on immune cells such as CD28 and LFA-1 (Grakoui et al., 1999; Malissen 1999).

Reverse signaling through CD137 ligand also occurs in monocytes. Immobilized but not soluble CD137 protein induces activation and proliferation and prolongs survival of peripheral monocytes (Langstein et al., 1998; Langstein et al., 1999a; Langstein et al., 1999b; Langstein

et al., 2000). It therefore seems possible that activation through CD137 ligand is functional in other APCs.

Ectopic CD137 expression provided protection from lysis by LAK cells while inhibition of constitutive CD137 expression enhanced susceptibility. This pattern was observed with all cells tested, which included human T and B cell lines and a largly undifferentiated myeloid cell line. Even COS and CHO cells, which are derived from a non-human primate and hamster, respectively, resisted lysyis by LAK cells after transfection with CD137 (not shown). The protective effect of CD137 was further confirmed using neutralizing anti-CD137 antibodies, which enhanced lysis by LAK cells.

CD137 has been shown to induce cell death by apoptosis in T cells (Schwarz et al., 1996; Michel at al., 1999). It was therefore reasonable to assume that CD137 expression would protect the target cells by inducing apoptosis in the LAK cells. This hypothesis could als be confirmed in this study. However, overall levels of apoptosis in the LAK cells after exposure to CD137-expressing target cells were low. Induction of apoptosis by CD137 in T cells is optimal after 2 – 3 days (Schwarz et al., 1996, Michel et al, 1999). But the LAK cells were exposed to CD137-expressing target cells only for the 4 h of the duration of the cytotoxicity assay, potentially explaining the low levels of apoptosis. It can however, not be ruled out that induction of T cell apoptosis plays a bigger role in vivo in CLL patients, where T cells encounter CD137-expressing tumor cells constantly.

The utilization of the inhibitory effects of TGF-β on immune functions by tumors has been documented previously (Akhurst and Derynck, 2001; Pasche, 2001). Schuler et al., (1999) demonstrated that autocrine TGF-β secretion by B-CLL cells inhibits T cell proliferation. We

found that increased CD137 expression correlates with enhanced TGF-β secretion secretion by the target cells while neutralization or inhibition of CD137 expression reduces constitutive expression of TGF-β.

The induction of TGF-β and the subsequent inhibition of LAK cell cytotoxicity complement earlier findings, which demonstrate that CD137 inhibits T cell proliferation and induces apoptosis in T cells (Schwarz et al., 1996; Michel et al., 1999). While the signals through CD137 are costimulatory for T cells, the signals through CD137 ligand seem to be inhibitory. An inhibitory effect of CD137 on T cells is also evident in vivo. Splenocytes of CD137 deficient mice respond with an increased proliferation to stimulation with mitogens or anti-CD3 (Kwon et al., 2002).

CD95 and CD95 ligand exert similar opposite effects on T cells. While the signals through CD95 induce apoptosis in CD8-positive T cells the signals through CD95 ligand are costimulatory (Suzuki and Fink, 1998; Desbartes et al., 1998).

Figure 8 illustrates a hypothesis of the physiological role of CD137 and its utilization by CLL cells. During the initial phase of an immune response antigen-specific T cells start to express CD137 after TCR engagement. CD137 ligand expressed by APC crosslinks CD137 delivering further activating signals to T cells (Fig. 7, upper panel). Costimulation by CD137 ligand or agonistic anti-CD137 antibodies enhance T cell activity in vitro and in vivo enabling tumor eradication in mice (Pollok et al., 1993; Schwarz et al., 1996; Melero et al., 1997). In the late phase of an immune response when the pathogen or antigen is cleared, APC will no longer provide growth and survival signals and the inhibitory activities of CD137 may gain the upper hand. Induction of TGF-β would provide an immediate and reversible way to reduce the

immune response. Induction of apoptosis by CD137 would require more time and terminate the immune response finally (Fig. 7, middle panel). Ectopic CD137 expression enables CLL cells to use these mechanisms to defend themselves against cytotoxic T cells (Fig. 7, bottom panel). Though antigen-primed APC should be present in CLL patients and deliver survival signals to the CLL-specific cytotoxic T cells the final outcome may be determined by the relative amounts of CD137 ligand on APC versus CD137 on CLL cells.

As outlined in Fig. 7 increasing the CD137/CD137 ligand ratio not only reduces T cell proliferation and survival. Increased CD137 expression enhances at the same time survival and proliferation of B cells, as demonstrated in this study and by previous work, respectively (Pollok et al., 1993, and Pauly 2002).

The observation that CD137 mRNA but not protein is inducible in primary B cells indicates a posttranscriptional control of CD137 expression. During the transformation process of B cells this posttranscriptional block is likely removed allowing malignant B cells to express CD137. CD137 then crosslinks CD137 ligand on the malignat B cells delivering survival signals and inducing TGF-β which protects the tumor cells from lysis by LAK cells.

In this study we have shown that through the ectopic expression of CD137 the tumor cells acquire the capabilities to inhibit LAK cell cytotoxicity via TGF-β secrection. These activities help tumor cells to escape from the host immune surveillance. The tumor cells retain the ability of primary B cells to become activated through CD137 ligand. This activity provides the basis for prolonged paracrine CD137-mediated survival.

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## FIGURE LEGENDS

Figure 1: CD137 is expressed by primary T but not by B cells

$5x10^6$ PBMC or selected lymphocytes in 1 ml medium were activated by 5 ng/ml PMA + 500 nM A23187 (PBMC, CD4+ T and CD8+ T) or by 10 µg/ml anti-CD40 + 100 ng/ml IL-4 (B) for 48 h. Expression of CD137 was analyzed by flow cytometry. White: anti-CD137; gray: isotype control.

Figure 2: CD137 is expressed by transformed B cells .

(A) B-CLL cells were activated with 10 µg/ml PHA for 24 h and (A) stained with the anti-CD137 antibody BBK-2 (dark line) or and isotype control (bright line) and analyzed by flow cytometry.

(B) Schematic representation of the percentage of CD137-positiver B cells from healthy donors (normal) and CLL patients. The number in parenthesis indicate the number of samples with an identical result.

(C) B-CLL cells were stained with and a FITC-labeled anti-CD19 antibody (green) and nuclei were stained with Hoechst (blue). In addition, the cells were stained with a RPE-labeled antibody for CD137 (red) (right panel) or a RPE-labeled isotype control antibody (left panel). Superimposition is shown in the top large photographs. Areas of colocalization of CD19 and CD137 appear orange or yellow. Single staining for CD19 or CD137 is shown in the smaller photographs at the bottom. Photographs were taken at a magnification of 400x.

(D) Serial frozen sections of a malignant anaplastic B cell lymphoma were stained with antibodies specific for CD137 (CD137), T cells (CD3), B cells (CD20) and an isotype control antibody (control). Staining with hematoxylin and eosin (HE) was used for visualization of the tissue. Photographs were taken at a magnification of 200x.

Figure 3: CD137 extends survival of B-CLL cells

$10^7$ B-CLL cells of patient TR were cultured on 5 μg/ml immobilized Fc or CD137-Fc protein. The numbers of live cells were determined at day 0, 3, 6, 9, 13, 17 and 20 and by trypan blue staining and are expressed as percentage live cells based on the number of live cells at the beginning of the experiment. Identical results were obtained in six independent experiments.

Figure 4: CD137 protects cells from lysis by LAKs

(A) PBMC were activated by 100 ng/ml of IL-2 for 3 days to generate LAK cells. Target cells were Jurkat, K562 and Raji cells, which had been transfected with a CD137 expression vector (RIS, black triangles), or a CD137 antisense expression vector (RIA, black circles), or an empty expression vector (RSV, open squares). Each data point represents the mean of 6 independent measurements. Identical results were obtained in three independent experiments.

(B) Modulation of CD137 expression on Raji and K562 cells by transfection with a CD137 expression vector (RSV-ILA-sen), or a CD137 antisense expression vector (RSV-ILA-antisen), or an empty expression vector (RSV). Expression of CD137 was detected by flow cytometry 48 h after transfection using anti-CD137 (bold lines) or isotype control antibodies (thin lines).

(C) COS cells were transfected with a CD137 expression vector (CIS, black symbols) or the empty vector (pcDNA3, open symbols), respectively. No antibody, anti-CD137 antibody (BBK-2) or an isotype control antibody (MOPC21) were added at a concentration of 5 μg/ml 6 h after transfection. The cells were used as targets in a cytotoxicity assay two days later (left panel).

Raji cells were grown for 16 h in the presence of 0.5, 1 or 5 μg/ml anti-CD137 antibody (BBK-2) before being used in a cytotoxicity assay. Cultures with no antibody and an isotype control antibody (MOPC21, 5 μg/ml) were used as controls. This experiment was repeated twice with identical results.

Figure 5: CD137 regulates expression of TGF-β by tumor cells.

Cells were transfected with a CD137 expression vector (RIS), or a CD137 antisense expression vector (RIA), or the empty vector (RSV). Cytokine concentrations of 24 h supernatants of $10^6$ transfected cells were determined in triplicates. Depicted are means ± standard deviations. This experiment was repeated four times with similar results.

Figure 6: Neutralization of TGF-β prevents CD137-mediated protection from LAK cell lysis.

Raji or K562 cells were transfected with a CD137 expression vector (RIS), or a CD137 antisense expression vector (RIA), or an empty expression vector (RSV). Neutralizing anti-TGF-β antibody or an isotype control antibody were added to a final concentration of 1 μg/ml. After 2 days the cells were washed and used as target cells in a cytotoxicity assay with activated PBMC (100 ng/ml of IL-2 for 3 days) as effector cells. Each condition was determined in hexaplicates. Identical results were obtained in three independent experiments.

Figure 7: Schematic representation of the proposed activities of CD137 expression on CLL cells. Top panel: During initiation of an immune response dendritic cells present antigen to T cells and provide T cell costimulation via CD137 Ligand and CD137, which are expressed on DC and T cells, respectively. Middle panel: After the antigen is cleared costimulation of T cells by DC ends. Paracrine induction of inhibitory cytokines and apoptosis in T cells by CD137 becomes predominant and contributes to the termination of the immune response.

Bottom panel: CLL cells express CD137 as a neoantigen and utilize its inhibitory activities to downregulate the host anti-tumor immune response.

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**Abstract**

CD137 (ILA, 4-1BB), a member of the TNF receptor family, and its ligand are expressed on T cells and antigen presenting cells, respectively. This receptor ligand system plays a role in T cell B cell interaction, and costimulates both cell types via bidirectional signal transduction. Immobilized CD137 protein enhances proliferation and immunoglobulin sysnthesis in primary B cells. Here we show that CD137 is expressed by chronic lymphocytic leukemia (CLL) cells but not by primary B cells. Immobilized CD137 protein prolongs survival of CLL cells in vitro, indicating that ectopic expression of CD137 allows CLL cells to imitate T cell B cell interactions and to provide each other with survival signals in a paracrine manner. Participation of CD137 in signaling is implied by its clustering into cell surface structures, which are compatible with microdomains. Expression of CD137 as a neoantigen provides CLL cells with a second selection advantage by protecting them from lysis by lymphokine activated killer (LAK) cells. Experimental modulation of CD137 expression on several model cell lines correlates with susceptibility to LAK lysis. Neutralization of CD137 by antibodies also reduces protection from lysis. Induction LAK cell apoptosis and TGF-β secretion by target cells were identified as underlying mechanisms.

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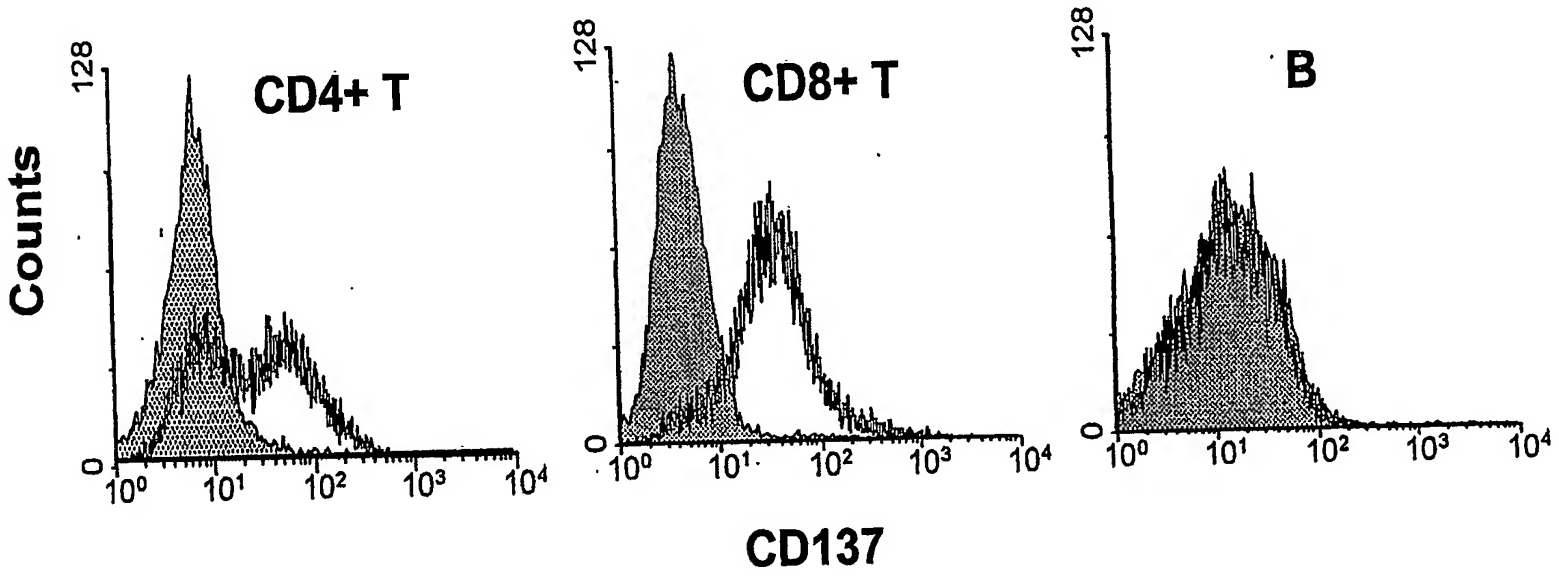



Figure 1

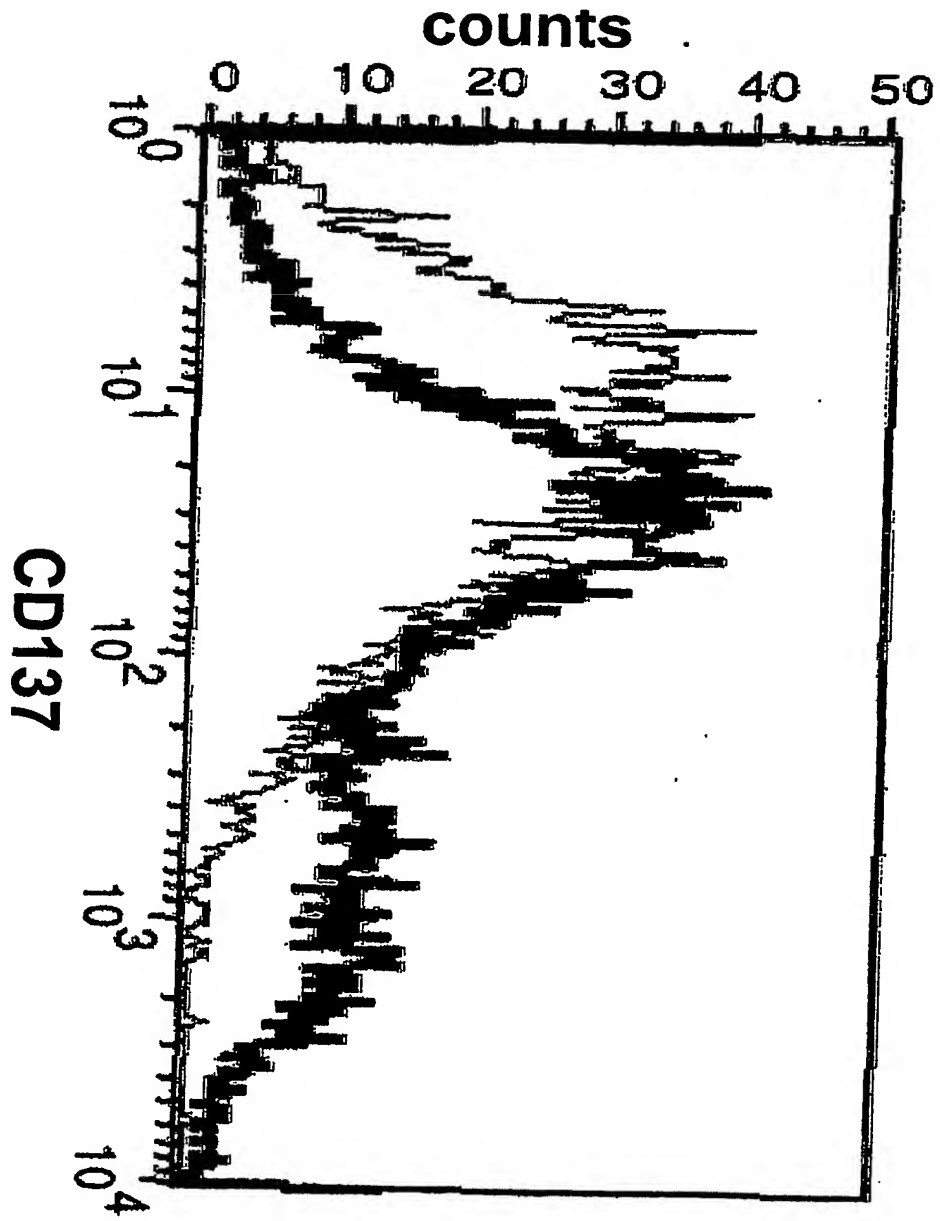
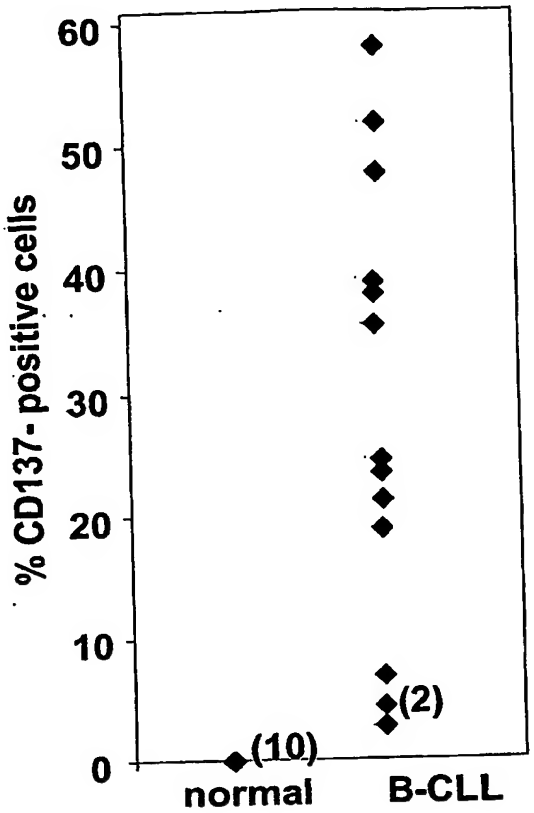



Figure 2A

Figure 2B



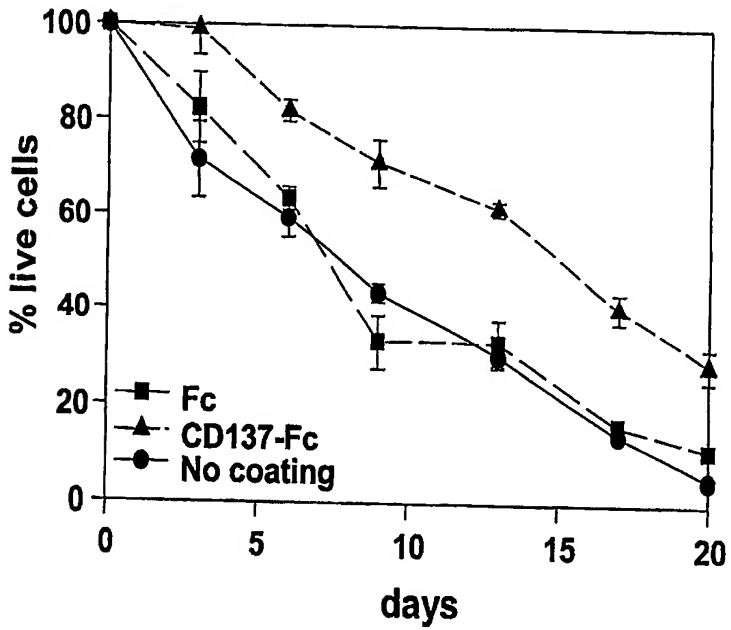
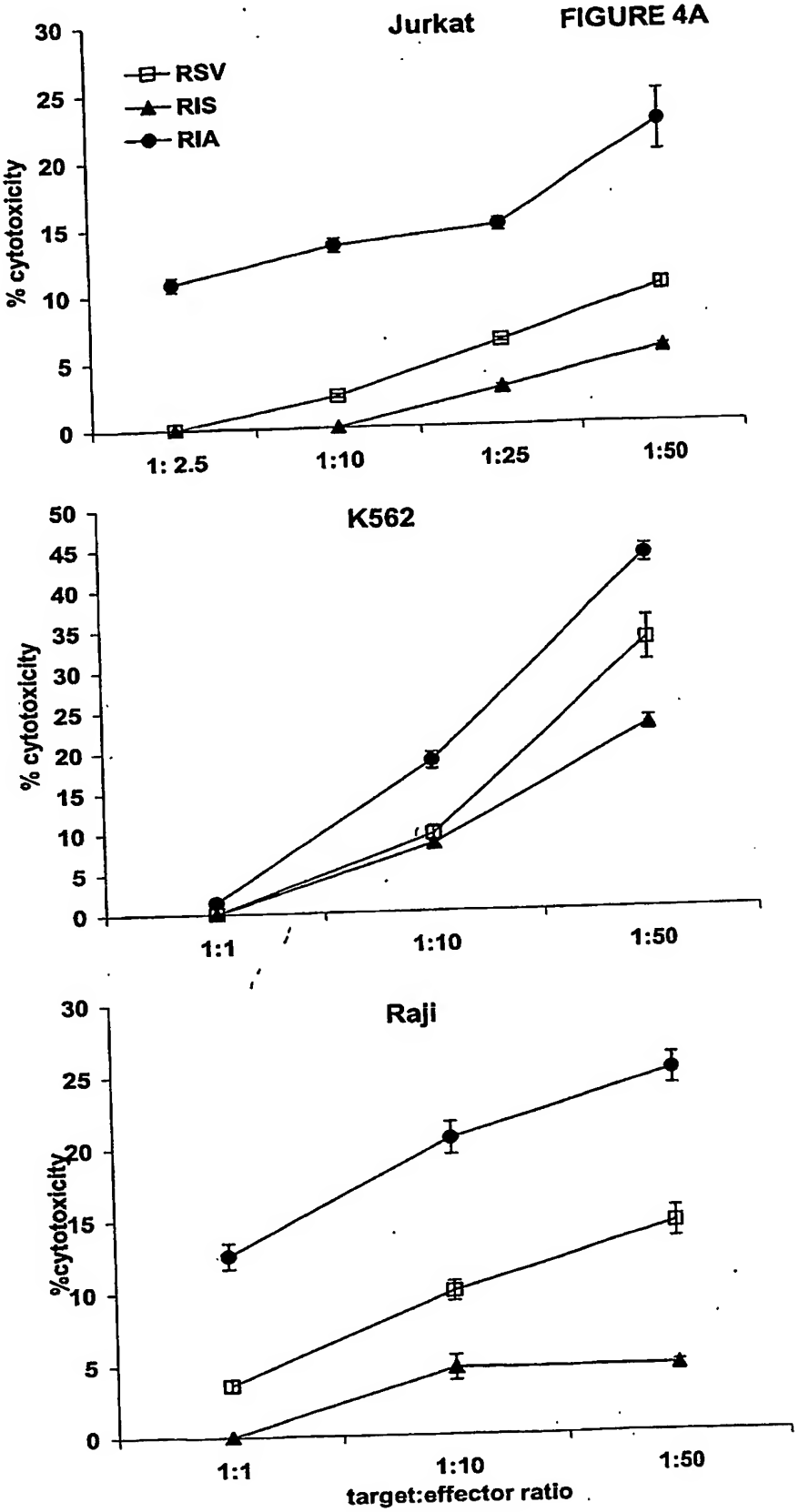
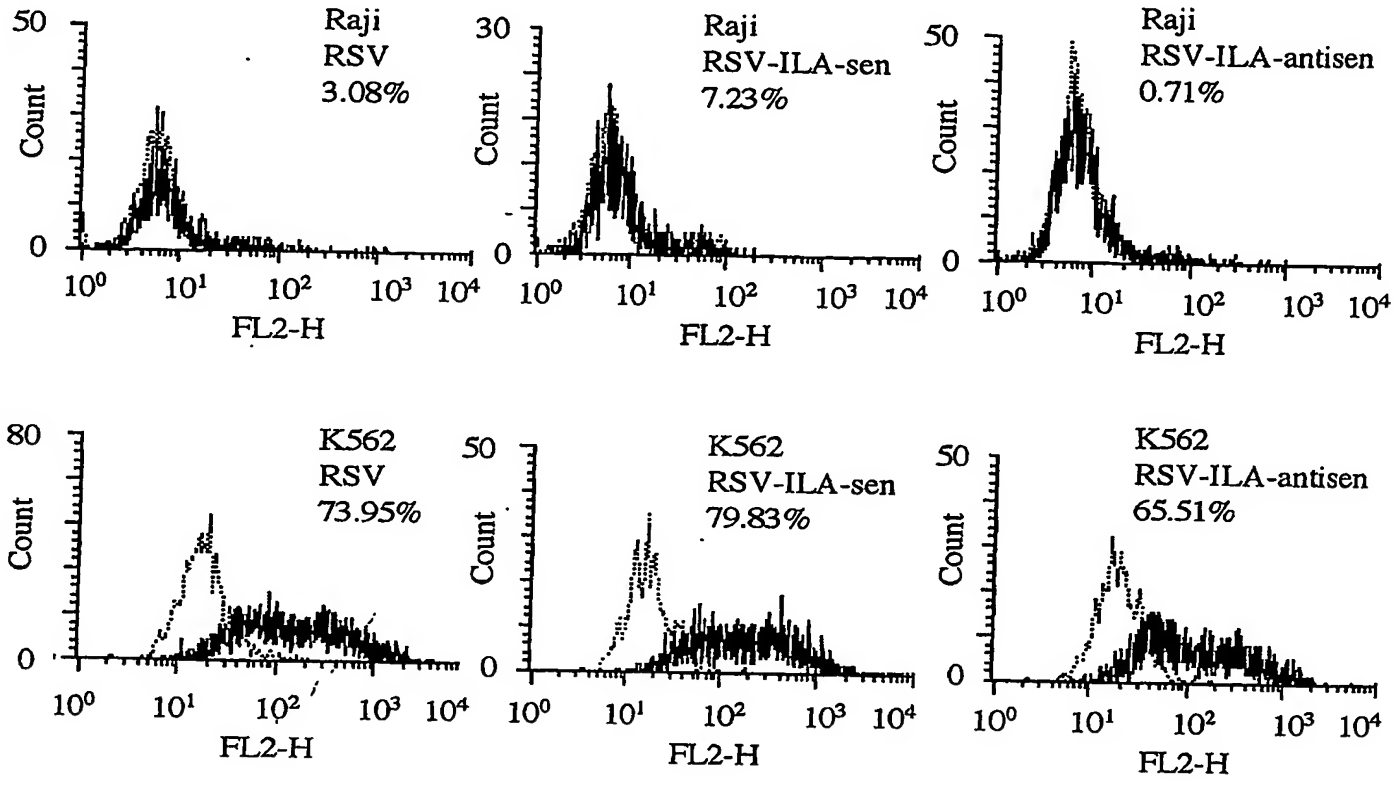

100
80
60
40
20
0
% live cells
Fc
CD137-Fc
No coating
0
5
10
15
20
days

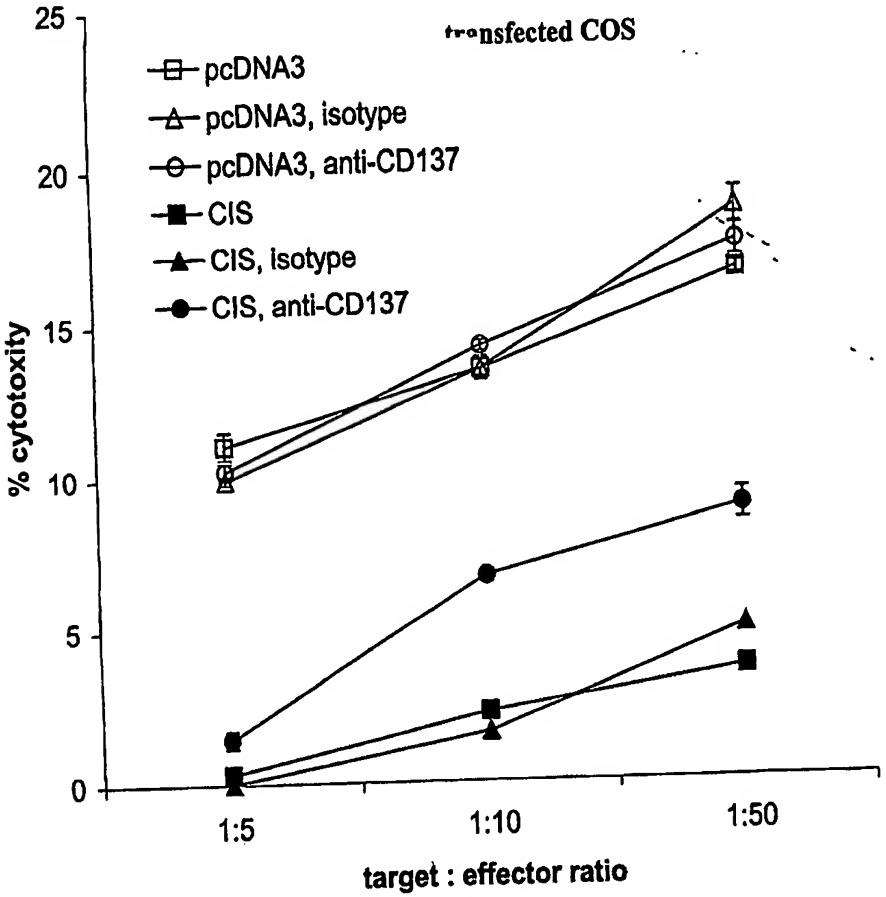
Figure 3

FIGURE 4A


Jurkat
RSV
RIS
RIA
% cytotoxicity
30
25
20
15
10
5
0
1: 2.5
1:10
1:25
1:50
K562
% cytotoxicity
50
45
40
35
30
25
20
15
10
5
0
1:1
1:10
1:50
Raji
%cytotoxicity
30
25
20
15
10
5
0
1:1
1:10
1:50
target:effector ratio

# Figure 4B


Raji
RSV
3.08%
Raji
RSV-ILA-sen
7.23%
Raji
RSV-ILA-antisen
0.71%
K562
RSV
73.95%
K562
RSV-ILA-sen
79.83%
K562
RSV-ILA-antisen
65.51%
Count
FL2-H
50
30
80
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$


transfected COS
pcDNA3
pcDNA3, isotype
pcDNA3, anti-CD137
CIS
CIS, isotype
CIS, anti-CD137
% cytotoxity
target : effector ratio
1:5
1:10
1:50
0
5
10
15
20
25

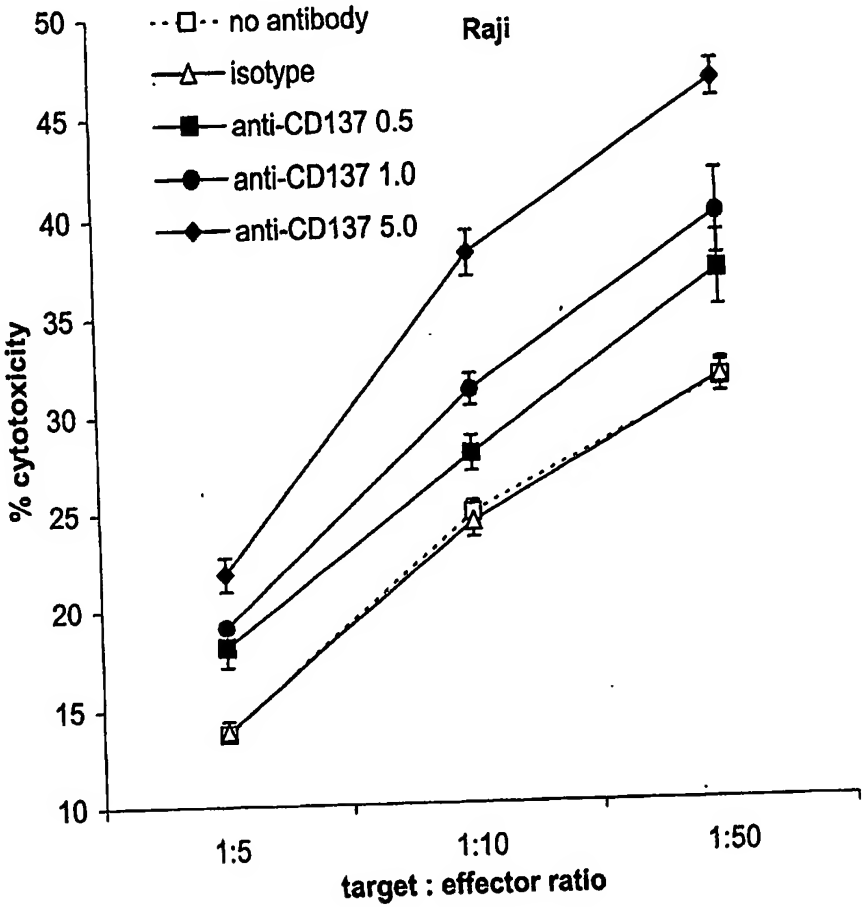

Raji
no antibody
isotype
anti-CD137 0.5
anti-CD137 1.0
anti-CD137 5.0
% cytotoxicity
target : effector ratio
1:5
1:10
1:50
10
15
20
25
30
35
40
45
50

FIGURE 4C

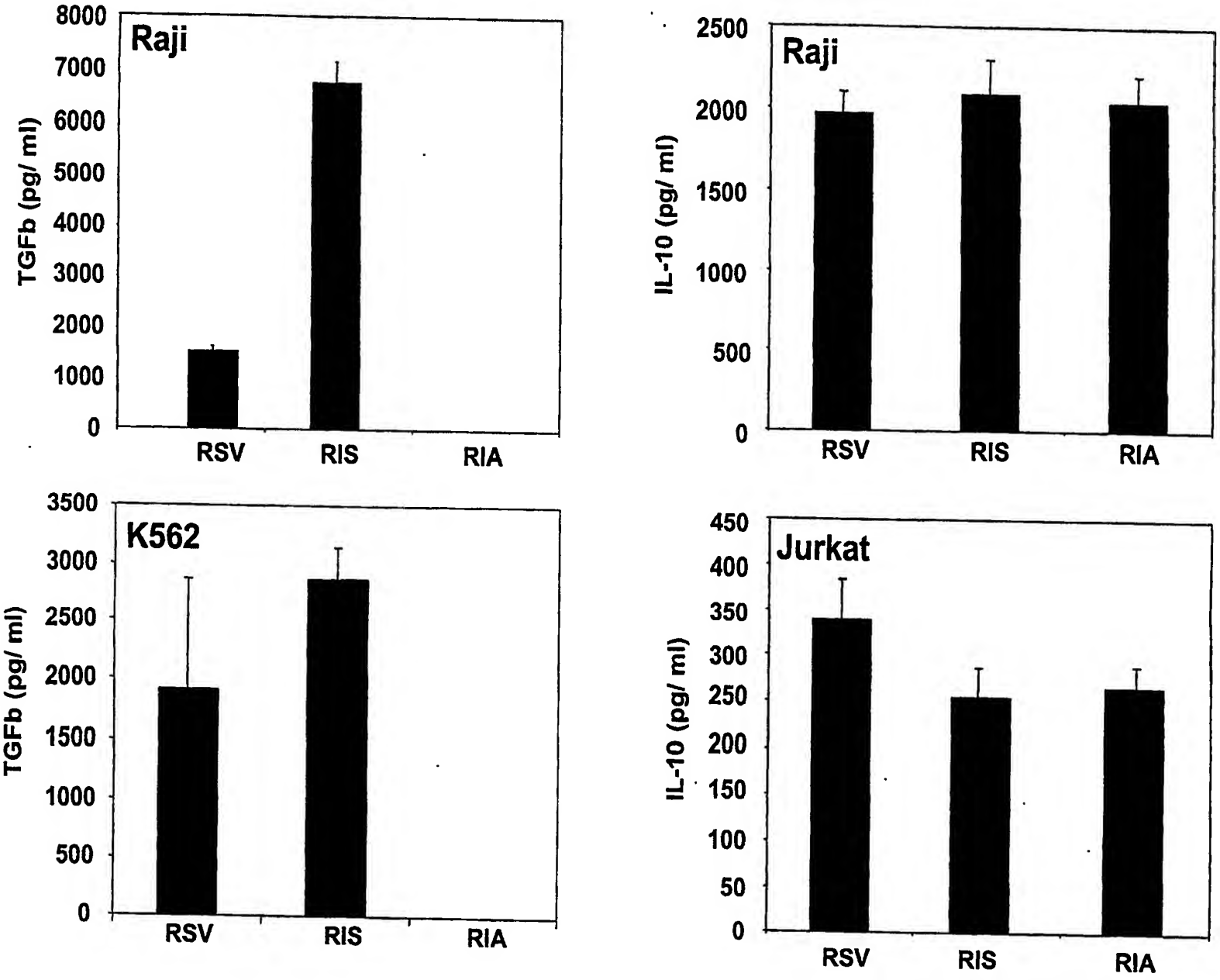
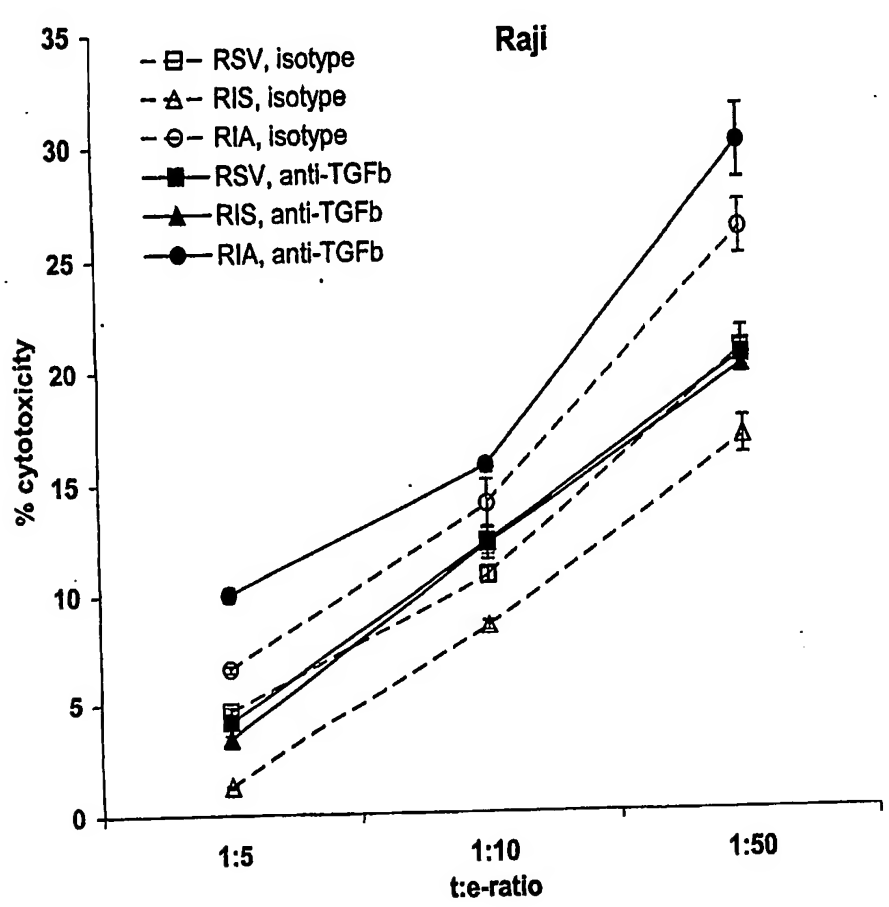

Raji
TGFb (pg/ ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
RSV
RIS
RIA
Raji
IL-10 (pg/ ml)
2500
2000
1500
1000
500
0
RSV
RIS
RIA
K562
TGFb (pg/ ml)
3500
3000
2500
2000
1500
1000
500
0
RSV
RIS
RIA
Jurkat
IL-10 (pg/ ml)
450
400
350
300
250
200
150
100
50
0
RSV
RIS
RIA

Figure 5


Raji
RSV, isotype
RIS, isotype
RIA, isotype
RSV, anti-TGFb
RIS, anti-TGFb
RIA, anti-TGFb
% cytotoxicity
0
5
10
15
20
25
30
35
1:5
1:10
1:50
t:e-ratio

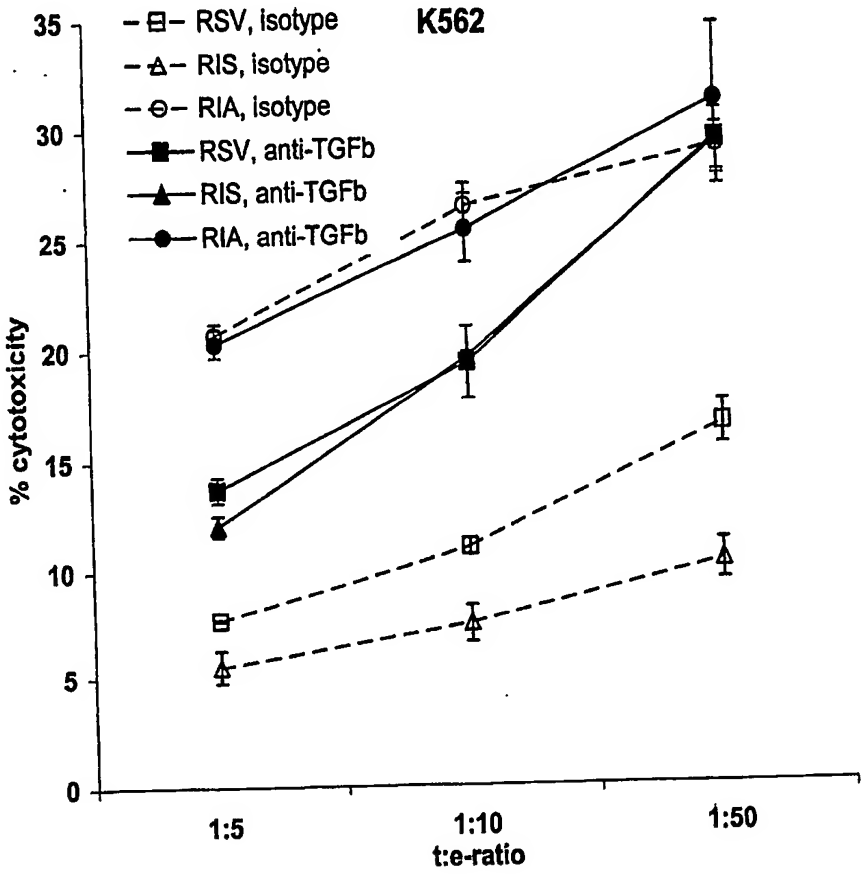
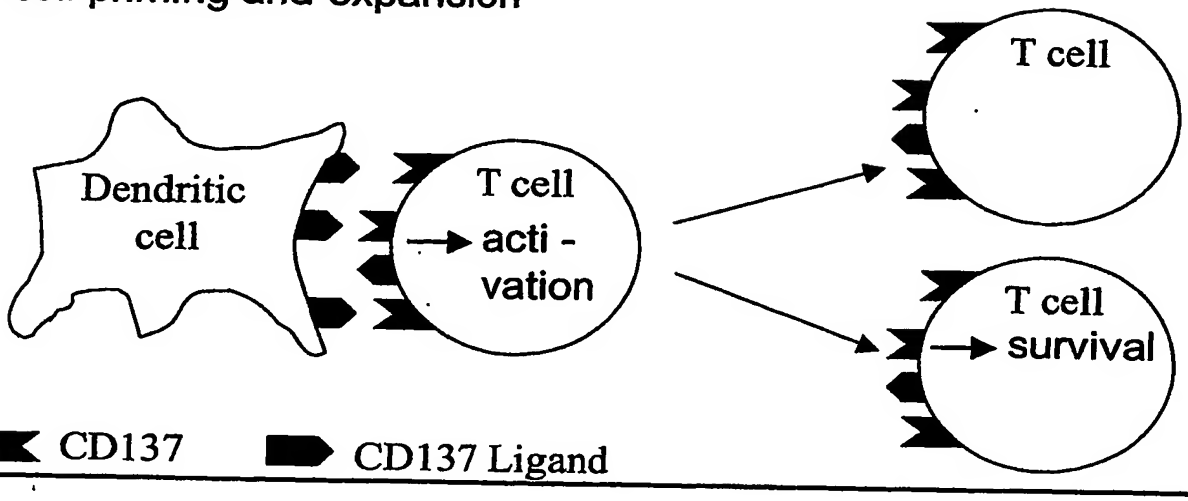

K562
RSV, isotype
RIS, isotype
RIA, isotype
RSV, anti-TGFb
RIS, anti-TGFb
RIA, anti-TGFb
% cytotoxicity
0
5
10
15
20
25
30
35
1:5
1:10
1:50
t:e-ratio

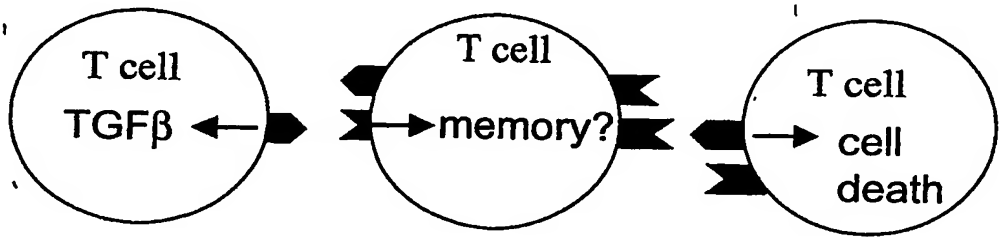
Figure 6

# Figure 7

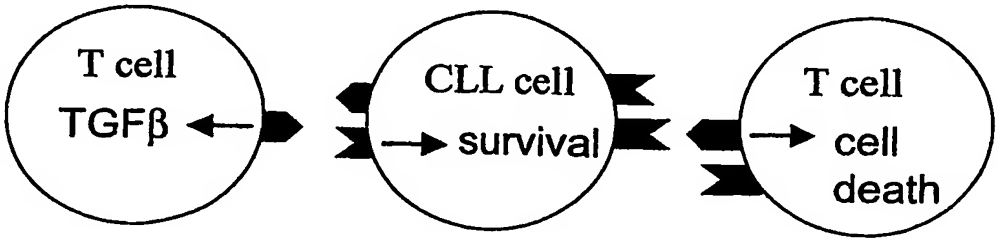
Normal situation in early immune response:
T cell priming and expansion




Normal situation in late immune response
Deletion of superfluous T cells




Pathologic situation in CLL:
Subversion of anti-CLL immune response



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